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Systematic Search for Variation in the Human Norepinephrine Transporter Gene: Identification of Five Naturally Occurring Missense Mutations and Study of Association With Major Psychiatric Disorders

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The complete coding region of the norepinephrine transporter (NET) gene was systematically screened for genetic variants in 137 unrelated individuals (including 46 probands with bipolar affective disorder and 45 schizophrenic probands, as well as 46 blood donors) using single-strand conformation analysis. We identified 13 DNA sequence variants, among them five missense substitutions. The missense substitutions Val69Ile, Thr99Ile, Val245Ile, Val449Ile, and Gly478Ser are located at putative transmembrane domains (TMD) 1, 2, 4, 9, and 10, respectively. The Thr99Ile substitution is at the 5th position of the putative leucine-zipper in TMD2. In a case-control study distribution of missense substitutions was found to be similar in 103 patients with bipolar affective disorder, in 228 schizophrenia patients and in 187 controls, indicating that presence of these variants is not causally related to major psychiatric diseases. The detection of a highly polymorphic silent 1287G/A polymorphism was utilized to demonstrate biallelic expression of the NET in adult human brain. © 1996 Wiley-Liss, Inc.

KEY WORDS: norepinephrine, noradrenaline, transporter, genetic variant, mutation analysis, schizophrenia, bipolar affective disorder, imprinting

INTRODUCTION

The human norepinephrine transporter (NET) is a Na^+- and Cl^--dependent, high affinity and substrate specific transporter belonging to the family of monoamine, GABA and amino-acid transporters [Uhl and Johnson, 1994]. These transporters reaccumulate and recycle released neurotransmitters into presynaptic terminals aiding termination of synaptic transmission. Even though Na^+- and Cl^--dependent transporters are not restricted to the nervous system, they seem to have evolved concomitantly with the emergence of neuronal cells [Nelson and Lill, 1994]. The monoamine transporters include the NET, the dopamine transporter (DAT) and the serotonin transporter (SERT). They have a similar general structure with 12 putative transmembrane domains (TMD) and a large extracellular loop between TMD 3 and 4 containing 2 to 4 potential glycosylation sites [Amara and Kuhar, 1993; Bönisch and Brüss, 1994]. NET reuptake activity is dynamically regulated by a variety of membrane and cytoplasmatic factors, including second messengers and hormones [King et al., 1992; Figlewicz et al., 1993]. The cDNA sequence of the human [Pacholczyk et al., 1991] and bovine [Lingen et al., 1994] NET gene has been reported. The gene encoding the human NET was assigned to the long arm of chromosome 16 by fluorescence in situ hybridization (FISH) [Brüss et al., 1993] and by linkage analysis [Gelernter et al., 1993]. It consists of 14 exons spanning 45 kilobases (kb) [Pörzgen et al., 1995]. The human NET is a 617 amino acid (aa) protein which shows 66% overall identity in aa sequence with the human DAT [Giros et al., 1992; Vandenbergh et al., 1992] and 48% identity with the human SERT [Lesch et al., 1993; Ramamoorthy et al., 1993].

A possible role of dysregulation of biogenic amines in neuropsychiatric diseases, including affective disorder and schizophrenia has been suggested. Monoamine transporters are the site of action of many antidepressants and addictive drugs (cocaine, amphetamine). The

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secondary tricyclic amines desipramine and nortriptyline belong to the most potent and selective inhibitors of the NET, whereas the tertiary tricycle amines (imipramine, amitriptyline) rather act through SERT inhibition [Hoffman, 1994]. Both short- and long-term desipramine treatment result in elevated NET mRNA in the locus coeruleus suggesting an increased expression and turnover in animal models [Szot et al., 1993].

Even though there are no consistently replicated findings on levels of biogenic amines in major depression, it has repeatedly been reported that some patients with major depression have increased norepinephrine plasma concentrations [Beckmann and Goodwin, 1980; Roy et al., 1985; Veith et al., 1994].

There is also evidence that the noradrenergic system could play a role in the etiology of schizophrenia. As dopamine, norepinephrine shows increased turnover during acute psychotic episodes [Gattaz et al., 1982; Linnoila et al., 1983]. Early relapse of the disease after neuroleptic withdrawal was predicted by an increased noradrenergic activity during treatment [van Kammen et al., 1994].

Given the possible dysregulation of biogenic amine systems in the major psychoses, an investigation of the NET gene as a candidate in bipolar affective disorder and schizophrenia was undertaken. In the present study we report results from a systematic mutation scan of the human NET gene in 137 unrelated individuals (including 46 bipolar affective and 45 schizophrenic probands, as well as 46 blood donors) using single-strand conformation analysis (SSCA) [Orita et al., 1989].

Thirteen nucleotide sequence variants were identified. Five of these were sequence changes that would result in protein alterations. In order to test for a possible contribution to the development of bipolar affective disorder and schizophrenia we determined mutation frequencies in large samples of unrelated patients and controls. In addition, we used the identification of a frequently occurring silent mutation to examine the question of a possible imprinting of NET in adult human brain.

MATERIAL AND METHODS

Individuals

The screening sample included 46 bipolar affective patients, 45 schizophrenic patients, and 46 blood donors as controls. In the screening sample 36 bipolars and 25 schizophrenics were derived from affected sib pairs and multiply affected pedigrees chosen at random prior to genotyping. The remaining subjects were recruited from inpatient and day hospital facilities.

For the association study a further 103 bipolar and 228 schizophrenic patients, as well as 187 blood donors were investigated for the presence of the five missense mutations. We calculated the statistical power of our case-control sample in the face of substantial genetic heterogeneity and polygenic inheritance. We considered a dominant mutation with a frequency of 1% in the general population leading to a relative risk of 5 (attributable risk 0.07). Under these assumptions our samples had a power of >0.80 and >0.90 to detect such an effect for bipolar affective disorder and schizophrenia, respectively ($\alpha = 0.05$).

All patients had been interviewed using the Schedule for Affective Disorders and Schizophrenia-Lifetime Version (SADS-L) [Endicott and Spitzer, 1978]. The diagnosis was assigned on the basis of the interview and medical records according to DSM III-R criteria [American Psychiatric Association, 1987]. All probands were unrelated and of German descent. Written informed consent was obtained from all patients participating in this study.

PCR Amplification of Genomic DNA

EDTA anticoagulated venous blood samples were collected from all probands. Leukocyte DNA was isolated by salting out with saturated NaCl solution [Miller et al., 1988].

Since the sensitivity of SSCA is optimal for PCR products of 150–300 base pairs (bp) length [Hayashi and Yandell, 1993], sets of primers were chosen resulting in fragment sizes from 186 bp to 288 bp (Table I). For exons 2–14 fragments encompassed the whole exon and adjacent exon-intron junctions. The 274 bp spanning exon 1 was screened using two overlapping fragments.

Standard PCR was carried out in 25 μl volume containing 40 ng genomic DNA, 10 pmol of each primer, 50 mM KCL, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatine, 200 μM of each dNTP, and 0.5 U Taq DNA polymerase (Life Technologies). The PCR amplification with primer sets 8, 9, 12, 14 was processed with additional 5% formamide and 10% glycerol. Samples were amplified in an UNO Thermoblock (Biometra). After an initial 5 min denaturation at 94°C, 35 temperature cycles were carried out consisting of 30 sec at 94°C, 30 sec at 57°C (except for fragment 4, for which annealing temperature was 58°C), and 30 sec at 72°C, followed by a final extension step of 5 min at 72°C.

Single-Strand Conformation Analysis (SSCA)

For non-radioactive SSCA 8μl of the PCR product were mixed with 12 μl formamide containing 0.0125% bromophenol blue and 0.75% Ficoll 400 in 1 × TBE, denatured for 5 min at 94°C and subsequently chilled on ice; 7μl of the product were loaded on 10% polyacrylamide (PAA) gels (acrylamide:bisacrylamide = 49:1; 110 × 130 × 1.0 mm, Multigel-Long/Biometra) containing 0.5 × TBE. PAA-gels were run for 12–15 h under two conditions: 6 V/cm at room temperature and 7 V/cm at +4°C. For exon 11, we used a modified procedure at 4°C with 10% PAA gels (acrylamide:bisacrylamide = 29:1) in 1× TBE running 8 h at 7 V/cm to get appropriate banding patterns. Bands were visualized by silverstaining [Budowle et al., 1991].

Cloning and Sequencing of PCR Products

PCR products from individuals displaying variant banding patterns in SSCA were cloned into pUC 18 SmaI/BAP vector (Pharmacia). Lysates of single colonies were used as templates for PCR with insert specific primers. SSCA of PCR products of different colonies allowed the identification of clones containing different alleles in heterozygous individuals. From selected colonies a hemibiotinylated PCR product was

TABLE I. Oligonucleotide Primers for Analysis of the Human Norepinephrine Gene

Primer sequence	Nucleotide position (5′ - 3′)[a]		Fragment size (bp)	Annealing temperature (°C)
14-F 5′-AAGTTCCTCTCGCCAGCC-3′	−24 - −7	5′ UTR	245	57
14-R 5′-ACTGCGAAGCCGACTACG-3′	221 - 204	Exon 1		
15-F 5′-CAAAACTGCGGAGCTGCT-3′	84 - 101	Exon 1	235	57
15-R 5′-GTGGACCTCCCAGATTCAAA-3′	+44 - +25	Intron 1		
1-F 5′-AGGGGTCTGTCAGGTCACAC-3′	−40 - −21	Intron 1	201	57
1-R 5′-ACCGTGACTTTCTCCCACC-3′	+29 - +11	Intron 2		
2-F 5′-TCCTACCTTACCCCCTGTCC-3′	−28 - −9	Intron 2	288	57
2-R 5′-GCACAAGGGGTCTGTGACTT-3′	+22 - +3	Intron 3		
3-F 5′-ATTTACCCTGGTCCCCTCC-3′	−35 - −17	Intron 3	222	57
3-R 5′-CCAAGGTTGATCACCCAAGT-3′	+48 - +29	Intron 4		
4-F 5′-GGGATTGGGGCCAGAG-3′	−49 - −34	Intron 4	214	58
4-R 5′-CCCAAGGCTTGGTGGTC-3′	+30 - +14	Intron 5		
5-F 5′-GGCTTTTGTCTGCTGGTTTC-3′	−22 - −3	Intron 5	186	57
5-R 5′-CACCCCACAAGAGTCAATCC-3′	+61 - +42	Intron 6		
6-F 5′-ACTTGACCTCACTGTGCTTCTTC-3′	−29 - −7	Intron 6	211	57
6-R 5′-TGGGCTTCTCAGCTCCC-3′	+56 - +40	Intron 7		
7-F 5′-CAGCCATTGATGAGGTCCTT-3′	−34 - −15	Intron 7	216	57
7-R 5′-CACTGTGTGTTGGGGAAGG-3′	+69 - +51	Intron 8		
8-F 5′-TCCAGGGAGACCCTAATTCC-3′	−49 - −30	Intron 8	241	57
8-R 5′-TTGACTTTATTGAAATGCGGC-3′	+63 - +43	Intron 9		
9-F 5′-CACAACAATCAGTTCCCACG-3′	−64 - −45	Intron 9	214	57
9-R 5′-CAGGATTCTAGGAGGACTGGG-3′	+50 - +30	Intron 10		
10-F 5′-AGAACCTCATGGGAGGACCT-3′	−73 - −54	Intron 10	245	57
10-R 5′-ATCCTCACCCAGCTCCATC-3′	+71 - +53	Intron 11		
11-F 5′-TGTCCTGTCTTCCTTTCTCTCC-3′	−41 - −20	Intron 11	246	57
11-R 5′-CTCCCACCCTGTTCCCCT-3′	+37 - +20	Intron 12		
12-F 5′-CAGGCTGCTAGAAGGGTGTC-3′	−62 - −43	Intron 12	251	57
12-R 5′-CTCACACTGGGTTTCTGGGT-3′	+117 - +98	Intron 13		
13-F 5′-CCCTTTCTGGGCCTCTGT-3′	−172 - −155	Intron 13	265	57
13-R 5′-TTGACGTAGCAGCGGATG-3′	1923 - 1906	3′ UTR		

[a]Exon sequences are numbered according to the published cDNA sequence [Pacholczyk et al., 1991], numbering of intron sequences is according to Pörzgen et al. [1995] and unpublished results.

generated using one biotylinated vector primer and one normal vector primer. The PCR product was incubated with streptavidine Dynabeads M-280 (Dynal, Ltd.) and magnetic beads were collected with a magnetic concentrator. After washing and denaturing both strands of DNA were sequenced by the dideoxy nucleotide chain termination method [Sanger et al., 1977] using Sequenase Version 2.0 Kit (US Biochemicals).

Restriction Enzyme Analysis

To verify genotypes and to determine the frequency of the respective variants, PCR-based restriction fragment length polymorphism (RFLP) assays were established for all polymorphic sites. For restriction analysis, original primer sets were used when the sequence variant altered a natural restriction site. In other cases we used mutagenic primers to introduce a base substitution adjacent to the sequence of interest, creating an artificial restriction site with only one allelic form (Table II). After amplification of genomic DNA 5μl of PCR product were digested with 5U of restriction enzyme BsiHKAI, 5U of DdeI, 4U of BstNI, 4U of NciI, 4U of BsaHI, 4U of RsaI, 2U of BsrDI, 0.8U of Tsp45I (Biolabs), or 1U of MnlI (Fermentas) according to the manufacturers' recommendations. Fragments were resolved on a 10% PAA gel (acrylamide:bisacrylamide = 49:1) containing 0.5 × TBE at 15 V/cm.

For detection of the exon 1 variant (Val69Ile) we performed a semi-nested PCR to reduce unspecific PCR products. First-round PCR was performed with primer pair 14F/15MR, second-round PCR with primer pair 15F/15MR.

cDNA-Preparation and Testing of Imprinting in Brain Tissue

To examine allelic expression of NET we studied brain tissue of the temporolateral cortex in two patients who had undergone surgery because of treatment-refractory epilepsy (code numbers TB740 and TB762). RNA extraction and cDNA preparation were as described previously [Cichon et al., 1995]. In brief, total RNA was extracted from 0.5 g brain tissue by acid guanidium thiocyanate-phenol-chloroform extraction [Chomczynski and Saachi, 1987]; first-strand cDNA was prepared using Superscript Preamplification System (Life Technologies). TB740 was identified to be heterozygous for the 1287G/A polymorphism (Thr429), TB762 was homozygous for the 1287G allele and served as a control. In order to show unambiguously that the PCR product is derived from cDNA and not from genomic DNA, the forward primer was located in exon 8 and the reverse primer was located in exon 9. The size of intron 8 is ~1.6 kb. To get appropriate fragment sizes after enzymatic digestion of the PCR-product, a mutagenic forward primer was created to destroy a constant Sau96I restriction site. 0.5 μl of cDNA was used for PCR amplification with primers 16F (5′-ATGCTCCTGGCGCTCGGCCTT-3′, nucleotide substitution is underlined) and 16R (5′-AGGGCGAGAAG-

TABLE II Mutagenic Primer Sequences to Introduce Appropriate Cleavage Sites for Specific Detection of Variants*

Primer sequence	Nucleotide position (5′ - 3′)		Fragment size (bp)	Annealing temperature (°C)
15-F 5′-CAAAACTGCGGAGCTGCT-3′	84 - 101	Exon 1	140	57
15M-R 5′-CCACTGCGAAGCCGAGTA-3′	223 - 207	Exon 1		
1M-F 5′-GCCTTCTTGATCCCGTGCA-3′	277 - 295	Exon 2	159	58
1-R 5′-ACCGTGACTTTCTCCCACC-3′	+29 - +11	Intron 2		
3-F 5′-ATTTACCCTGGTCCCTCC-3′	−35 - −17	Intron 3	144	57
3M-R 5′-GCTAAAATACAAGACGGTGA-3′	753 - 734	Exon 4		
7M-F 5′-CAGCCATTGATGAGGTCATTG-3′	−34 - −14	Intron 7	216	57
7-R 5′-CACTGTGTGTTGGGGAAGG-3′	+69 - +51	Intron 8		
10-F 5′-AGAACCTCATGGGAGGACCT-3′	−73 - −54	Intron 10	214	55
10M-R 5′-CCCTCCCCACATGCCGG-3′	+40 - +20	Intron 11		
12M-F 5′-AGACTGGCCTATGGCATGAC-3′	1759 - 1778	Exon 13	189	57
12-R 5′-CTCACACTGGGTTTCTGGGT-3′	+117 - +96	Intron 13		

*__indicates nucleotide substitution in the mutagenic primers.

GAAAGTGCTG-3′) under standard conditions (see above) except that the annealing temperature was set at 62°C. PCR resulted in a 142 bp PCR fragment. Depending on the absence or presence of the polymorphic Sau96I site, either a fragment of 77 bp (1287A) or two fragments of 56 and 21 bp (1287G) are produced. Cleavage in a nonpolymorphic Sau96I site produces a constant fragment of 65 bp.

Statistical Analysis

Fisher's exact test was carried out to test the null hypothesis of no difference in the allele and genotype frequency distribution for patients and controls.

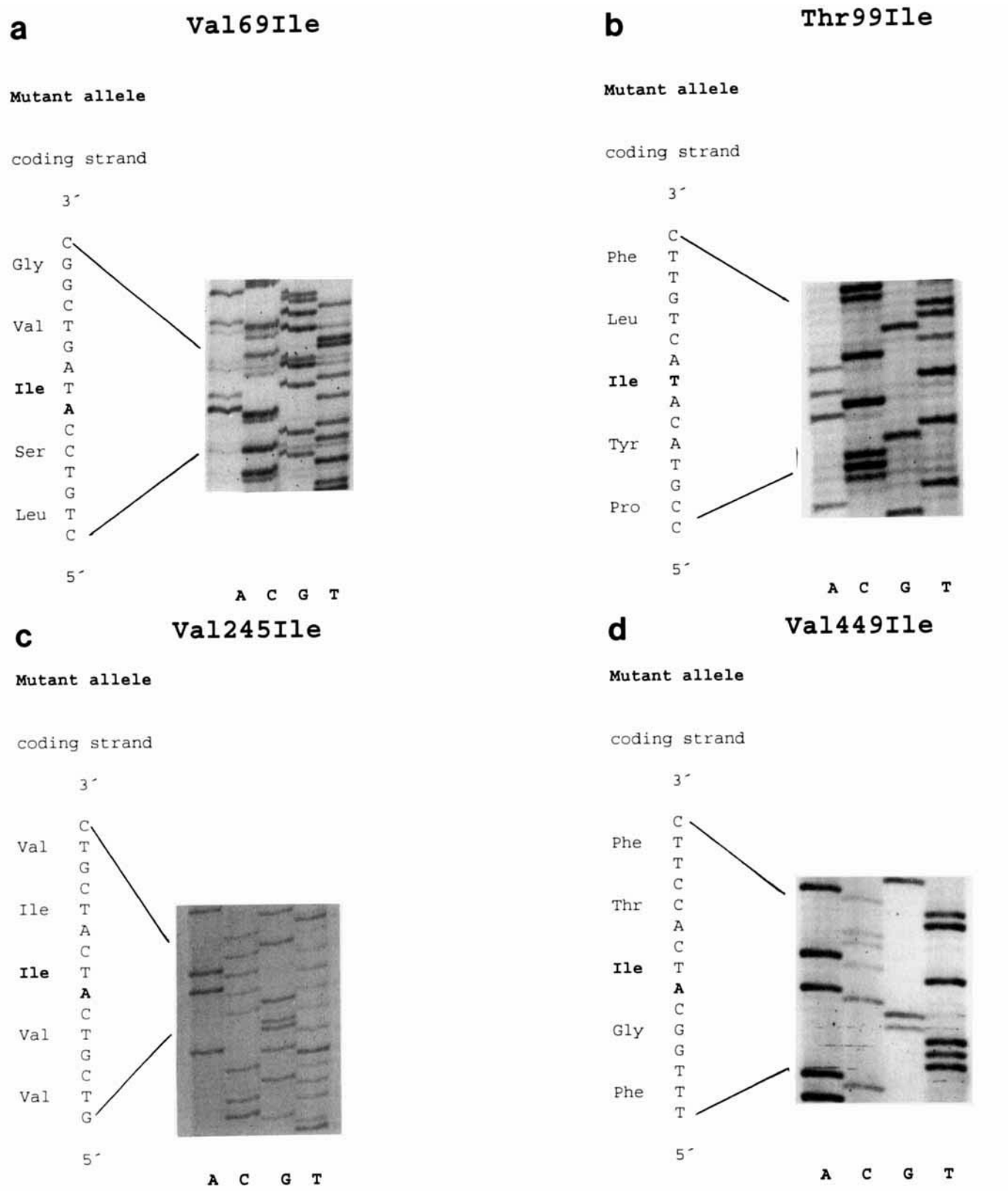
RESULTS

Screening for Mutations

For mutation analysis, the whole coding region of the human NET gene consisting of 14 exons and adjacent exon-intron-boundaries was systematically screened for nucleotide variation. A total of 137 individuals (including 46 bipolar patients, 45 schizophrenics, and 46 controls) were investigated. Among the 274 NET alleles 13 sequence changes were identified (Table III). Five sequence variants resulted in amino-acid substitutions (Val69Ile, Thr99Ile, Val245Ile, Val449Ile, and Gly478Ser) (Fig. 1), three were silent mutations (955T/C, 1287G/A, and 1779G/A), and five were muta-

TABLE III. Structural Variants and Polymorphisms in the Human Norepinephrine Transporter

Exon/ intron	Location (nucleotide position)	Sequence change	Protein variant	Restriction-enzyme	Primer pair	PCR-product (bp)	Allele	Allele fragment size (bp)
Exon 1	205	G→A	Val69Ile	RsaI	15-F/15M-R	140	A1	140
							A2	123 + 17
Exon 2	296	C→T	Thr99Ile	BsiHKAI	1M-F/1-R	159	B1	159
							B2	140 + 19
Exon 4	733	G→A		Tsp45I	3-F/3M-R	144	C1	144
							C2	123 + 21
Intron 5	918 + 11	A→G		DdeI	4-F/4-R	214	D1	214
							D2	192 + 22
Exon 6	955	T→C		BstNI	5-F/5-R	187	E1	164 + 23
							E2	105 + 59 + 23
Intron 7	1148 − 13	C→A		BsrDI	7M-F/7-R	216	F1	216
							F2	200 + 16
Exon 9	1287	G→A		Sau96I	8-F/8-R	241	G1	113 + 76 + 31 + 21
							G2	113 + 97 + 31
Exon 9	1345	G→A	Val449Ile	Tsp45I	8-F/8-R	241	H1	200 + 41
							H2	133 + 67 + 41
Intron 9	1389 + 9	G→A		MnlI	8-F/8-R	241	I1	156 + 42 + 18 + 13 + 7 + 4 + 1
							I2	124 + 42 + 32 + 18 + 13 + 7 + 4 + 1
Exon 10	1432	G→A	Gly478Ser	BsiHKAI	9-F/9-R	214	J1	214
							J2	110 + 104
Intron 11	1590 + 23	T→C		NciI	10-F/10M-R	214	K1	122 + 92
							K2	122 + 76 + 16
Exon 13	1779	G→A		BsaHI	12M-F/12-R	189	L1	189
							L2	169 + 20
Intron 13	1830 + 66	T→C		BsiHKAI	12-F/12-R	251	M1	154 + 97
							M2	104 + 97 + 50


a
Val69Ile
Mutant allele
coding strand
3´
Gly
Val
Ile
Ser
Leu
5´
A C G T
b
Thr99Ile
Mutant allele
coding strand
3´
Phe
Leu
Ile
Tyr
Pro
5´
A C G T
c
Val245Ile
Mutant allele
coding strand
3´
Val
Ile
Ile
Val
Val
5´
A C G T
d
Val449Ile
Mutant allele
coding strand
3´
Phe
Thr
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Gly
Phe
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Fig. 1.

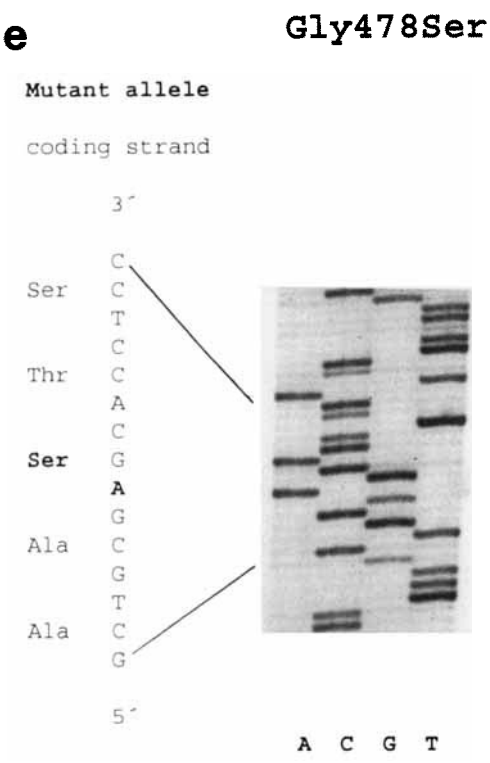

Fig. 1. DNA sequence analysis revealing the five missense substitutions in the human norepinephrine transporter gene. Coding strand of the minor allele is shown for Val69Ile (**a**) Thr99Ile (**b**) Val245Ile (**c**) Val449Ile (**d**) and Gly478Ser (**e**).

tions in flanking intron sequences (introns 5, 7, 9, 11, and 13).

Frequency of Structural Variants and Polymorphisms

Allele frequencies for the 13 polymorphisms observed in the screening sample and for the 5 coding variants in a larger case-control sample are given in Table IV. Genotype distribution (data not shown) and allele frequency statistics in the different diagnostic groups revealed no significant differences. Missense substitutions Val69Ile, Thr99Ile, and Val245Ile were found in similar frequencies in all groups. Val449Ile was observed in a single schizophrenic patient who had no family history of major psychiatric disease. Gly478Ser was found in a single control proband. The only individual homozygous for a missense substitution (Thr99Ile) found in our sample was a blood donor on whose physical and psychiatric conditions no information was available. Absolute linkage disequilibrium was found between the polymorphisms 1287G/A (Exon 9) and 1389+9G/A (Intron 9) which are only 110 bp apart.

Imprinting of the Human Norepinephrine Transporter

On the genomic level individual TB740 was identified to be heterozygous for the 1287G/A polymorphism. Individual TB762 was homozygous and served as a control. Following extraction of total RNA from tempolateral cortex first-strand cDNA was prepared by reverse transcription. Using cDNA as PCR-template a 142 bp fragment was amplified using primers spanning intron 8 (~1.6 kb) to ensure that the product was derived from cDNA and not from genomic DNA. The restriction pattern of the PCR-product unambiguously shows a pattern of heterozygosity in individual TB740 (Fig. 2) indicating biallelic expression of the NET gene.

DISCUSSION

In the present examination of the human NET gene in 46 patients with bipolar affective disorder, 45 schizophrenic patients and 46 controls, 13 nucleotide sequence variants were detected. These consisted of five missense substitutions, three silent mutations and five intron variants. The three silent mutations are probably without functional consequences on transporter function since they should not introduce aberrant splice sites according to their Senapathy score [Shapiro and Senapathy, 1987]. Similarly, the five intron variants do not affect known splice sites.

The amino acid substitutions Val69Ile, Thr99Ile, Val245Ile, Val449Ile, and Gly478Ser are located at putative TMD 1, 2, 4, 9, and 10, respectively (Fig. 3). The hydrophobic domains 1, 2, 4–8 are the most conserved domains within the neurotransmitter transporter family [Amara and Kuhar, 1993].

The Val69Ile replacement at the 5th position of TMD1 is localized in a strongly conserved region. Val69 is found in bovine NET and in all human monoamine transporters. Val69 is adjacent to a conserved asparagine acid (Asp75) which seems to play a key role in determining substrate uptake. Site directed mutagenesis of this Asp residue in DAT has been shown to reduce apparent affinity for dopamine by a factor of 3–6 but also dramatically impairs dopamine uptake [Kitayama et al., 1992]. There is evidence from structure-function analyses using chimeric catecholamine transporters that the Asp residue is not directly responsible for functional differences between NET and DAT (Asp75 is conserved in NET), but determinants in close proximity contribute essentially to specific substrate affinity via hydrophobic and/or van der Waals bonding [Buck and Amara, 1994].

Thr99Ile is located in the 5th position of a 22 aa spanning putative leucine-zipper in TMD 2 [Pacholczyk et al., 1991]. The threonine residue is conserved in bovine NET and human SERT. To date there is little definite information regarding the specific function of leucine-zippers in membrane-spanning domains of neurotransmitter transporters. Substitution of leucine in voltage-dependent K^+ ion channels was shown to alter the conformational stability during gating and assembly, and point mutations at non-leucine residues resulted in alterations of the voltage dependence for activation [Auld et al., 1990; McCormack et al., 1993]. In tyrosine hydroxylase deletion of the leucine zipper motif or mutation of a leucine residue within the zipper led subsequently to a drastically reduction in specific activity and to conversion of the tetrameric to dimeric assembly [Vrana et al., 1994]. Since the Thr99Ile variant changes aa polarity from hydrophobic to hydrophilic, it may alter

TABLE IV. Distribution of Minor Alleles in Patient and Control Samples*

Allele	Screening sample				Association sample			
	Controls (n = 92)	Schizophrenics (n = 90)	Bipolars (n = 92)	P value	Controls (n = 374)	Schizophrenics (n = 456)	Bipolars (n = 206)	P value
A1 [Ile69]	1 [1.1]	1 [1.1]	1 [1.1]	1.00[a] 1.00[b]	1 [0.3]	0 [0.0]	0 [0.0]	0.45[a] 1.00[b]
B1 [Ile99]	2 [2.2]	1 [1.1]	2 [2.2]	1.00[a] 1.00[b]	9 [2.4]	9 [2.0]	1 [0.5]	0.81[a] 0.11[b]
C1 [Ile245]	1 [1.1]	0 [0.0]	1 [1.1]	1.00[a] 1.00[b]	0 [0.0]	3 [0.7]	1 [0.5]	0.26[a] 0.36[b]
D1	5 [5.4]	5 [5.6]	6 [6.5]	1.00[a] 1.00[b]				
E2	3 [3.3]	3 [3.3]	0 [0.0]	1.00[a] 0.25[b]				
F2	37 [40.2]	28 [31.1]	34 [37.0]	0.22[a] 0.76[b]				
G1	32 [34.8]	28 [31.1]	31 [33.7]	0.64[a] 1.00[b]				
H1 [Ile449]	0 [0.0]	1 [1.1]	0 [0.0]	0.50[a]	0 [0.0]	0 [0.0]	0 [0.0]	
I1	32 [34.8]	28 [31.1]	31 [33.7]	0.64[a] 1.00[b]				
J2 [Ser478]	1 [1.1]	0 [0.0]	0 [0.0]	1.00[a] 1.00[b]	0 [0.0]	0 [0.0]	0 [0.0]	
K2	20 [21.8]	15 [16.7]	11 [12.0]	0.45[a] 0.11[b]				
L1	0 [0.0]	3 [3.3]	3 [3.3]	0.12[a] 0.25[b]				
M2	33 [35.9]	33 [36.7]	22 [23.9]	1.00[a] 0.11[b]				

*Frequencies are given in absolute numbers and percentage [ ]; n = number of alleles. Fisher's exact test (two-tailed).
[a]Controls vs. schizophrenics.
[b]Controls vs. bipolars.

leucine-zipper function and thereby affect assembling stability during activation or stability of transporter complexes during folding or insertion processes.

Val245Ile is located in a non-conserved region of TMD 4. The valine residue is found in the bovine NET, but not in other human monoamine transporter proteins, whereas the isoleucine residue is found in mammalian DATs. Interestingly, Val245Ile is close to the analogous locus of the only known variant of the human SERT (Leu255Met) [DiBella et al., 1995] which might point to an increased genetic variability of TMD4 in neurotransmitter-transporters.

Two missense variants (Val449Ile and Gly478Ser) were found in the putative transmembrane domains 9 and 10 which are defined as determinants for substrate affinity and stereoselectivity [Giros et al., 1994]. The valine residue in position 449 is conserved in bovine NET and human SERT. The Gly478Ser change occurs at a highly conserved generic residue; it is identical in all monoamine transporters in all species known. Gly478Ser introduces a second serine residue at the extracellular side of TMD10. Serine residues are proposed to act as hydrogen bond donor for interaction with substrates, and thus, Gly478Ser may be involved in dynamically regulated functions of the norepinephrine transporter.

Based on the findings of disturbances of the noradrenergic system in affective disorder and schizophrenia we hypothesized that the identified missense substitutions might well be involved in the genetic predisposition to these diseases. However, case-control analyses using large samples of patients with bipolar affective disorder and schizophrenia failed to reveal any significant association of the missense substitutions with the disease. Val69Ile, Thr99Ile, and Val245Ile were observed in similar frequencies in patients and controls. Gly478Ser was observed in a single control proband. Only the Val449Ile substitution was found in a schizophrenic patient and not in controls. However, there was no family history of psychiatric disease in the patient's family making this mutation an unlikely cause of an inherited form of schizophrenia. These results indicate that none of the identified protein variants contributes significantly to the development of major psychiatric disease. In accordance with our findings, negative lod scores were obtained in six multigenerational families in which bipolar affective disorder segregates using a TaqI polymorphism at the NET locus [Hadley et al., 1995]. However, the linkage approach is more susceptible to an underlying heterogeneity than association studies and is mainly designed to detect major gene effects. Genes with a relatively small although significant contribution to the disease may escape detection by applying a linkage approach but might well be detected in a conventional case-control association study [Greenberg, 1993; Nöthen et al., 1993]. Therefore, a negative linkage finding would not be incompatible with a polygenic action or with a gene which is responsible for the disease in only a subgroup of patients.

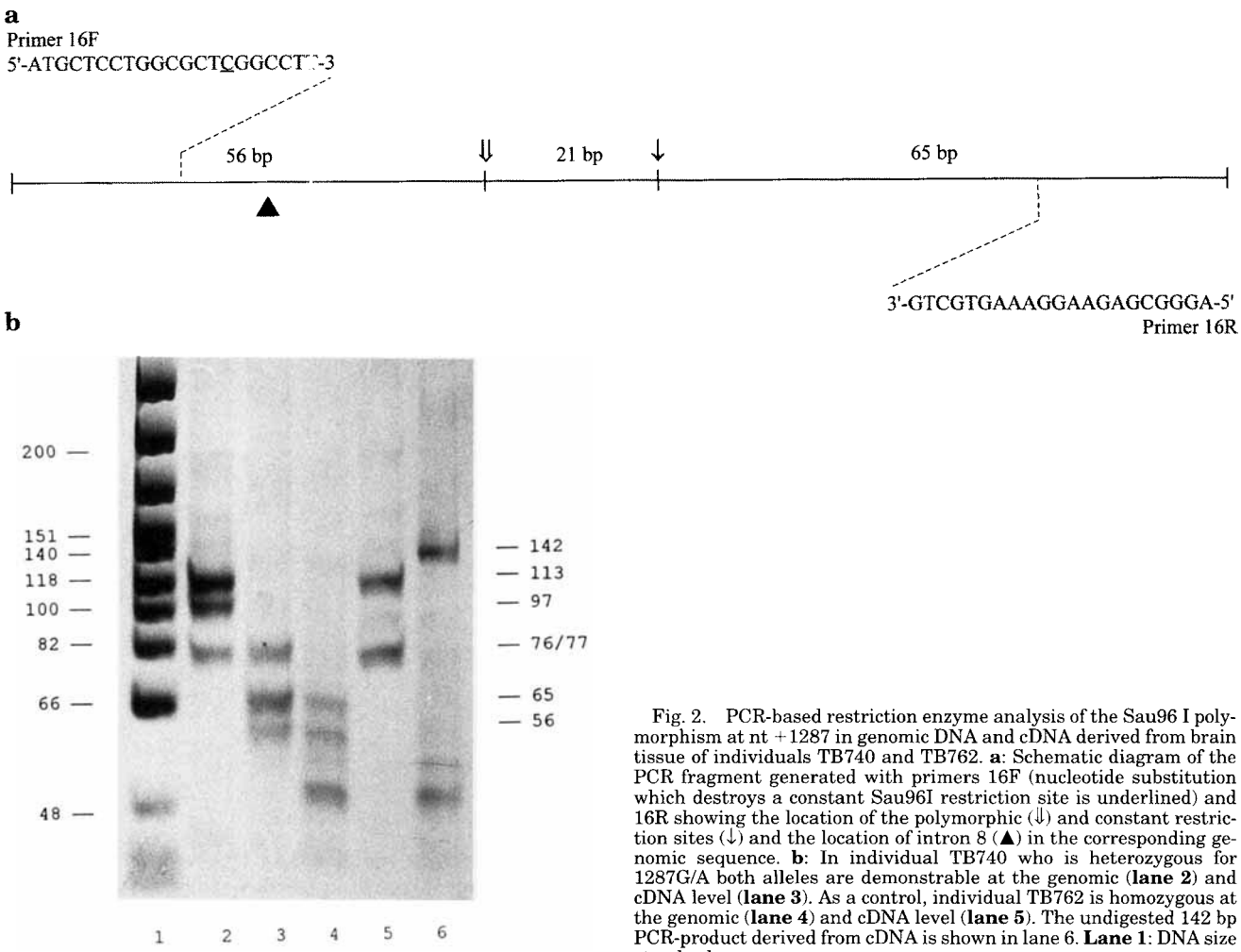

Fig. 2. PCR-based restriction enzyme analysis of the Sau96 I polymorphism at nt +1287 in genomic DNA and cDNA derived from brain tissue of individuals TB740 and TB762. **a**: Schematic diagram of the PCR fragment generated with primers 16F (nucleotide substitution which destroys a constant Sau96I restriction site is underlined) and 16R showing the location of the polymorphic (⇓) and constant restriction sites (↓) and the location of intron 8 (▲) in the corresponding genomic sequence. **b**: In individual TB740 who is heterozygous for 1287G/A both alleles are demonstrable at the genomic (**lane 2**) and cDNA level (**lane 3**). As a control, individual TB762 is homozygous at the genomic (**lane 4**) and cDNA level (**lane 5**). The undigested 142 bp PCR-product derived from cDNA is shown in lane 6. **Lane 1**: DNA size standard.

Our sample size of 45 bipolar and 46 schizophrenic patients, respectively, would have allowed a 90% chance of finding a mutation if the frequency of the mutation was at least 5% in the respective patient sample. However, if the transporter is defective in rare cases, we may have missed such individuals. Furthermore, it cannot be excluded that mutations in regulatory sequences such as those in promoter or enhancer sequences may be involved in a subset of patients. Finally, there remains the possibility that we have missed a mutation by relying on SSCA as a mutation screening procedure because the sensitivity of SSCA is not 100% [Hayashi and Yandell, 1993]. This possibility has been reduced by performing SSCA under two partly different conditions. In fact, nine mutations showed altered migration only under one of the two conditions applied. However, the existence of undetected variants cannot be completely excluded.

Genomic imprinting is a mechanism in mammals whereby alleles of a gene are differentially expressed depending on their parental origin. It has been suggested as one of the mechanisms to account for the apparent non-Mendelian inheritance pattern which is observed in most of the common neuropsychiatric diseases [Hall, 1990]. If the NET gene was imprinted this would have important consequences for the evaluation of linkage and association studies in these diseases. The phenotypic expression of the NET variants would then depend on maternal or paternal inheritance of the allele so that only the expressed allele would have to be taken into consideration. We investigated the expression of NET in brain tissue of two patients who had undergone surgery because of treatment-refractory epilepsy. The 1287G/A polymorphism was utilized to distinguish between expression of the maternal and paternal allele. In a heterozygous patient we could demonstrate biallelic expression of the NET gene indicating that NET is not imprinted in adult human brain. However, it is important to stress that imprinting can be a tissue specific process [DeChiara et al., 1991; Jinno et al., 1994]. Insofar as only neuronal tissue was analyzed, the conclusions need to be restricted to this type of tissue. Imprinting can also be dependent on the developmental stage [DeChiara et al., 1991]. Therefore, the possibility of imprinting in early phases of development cannot be ruled out from the present study.

In conclusion, this is the first demonstration of naturally occurring structural variability in human NET.

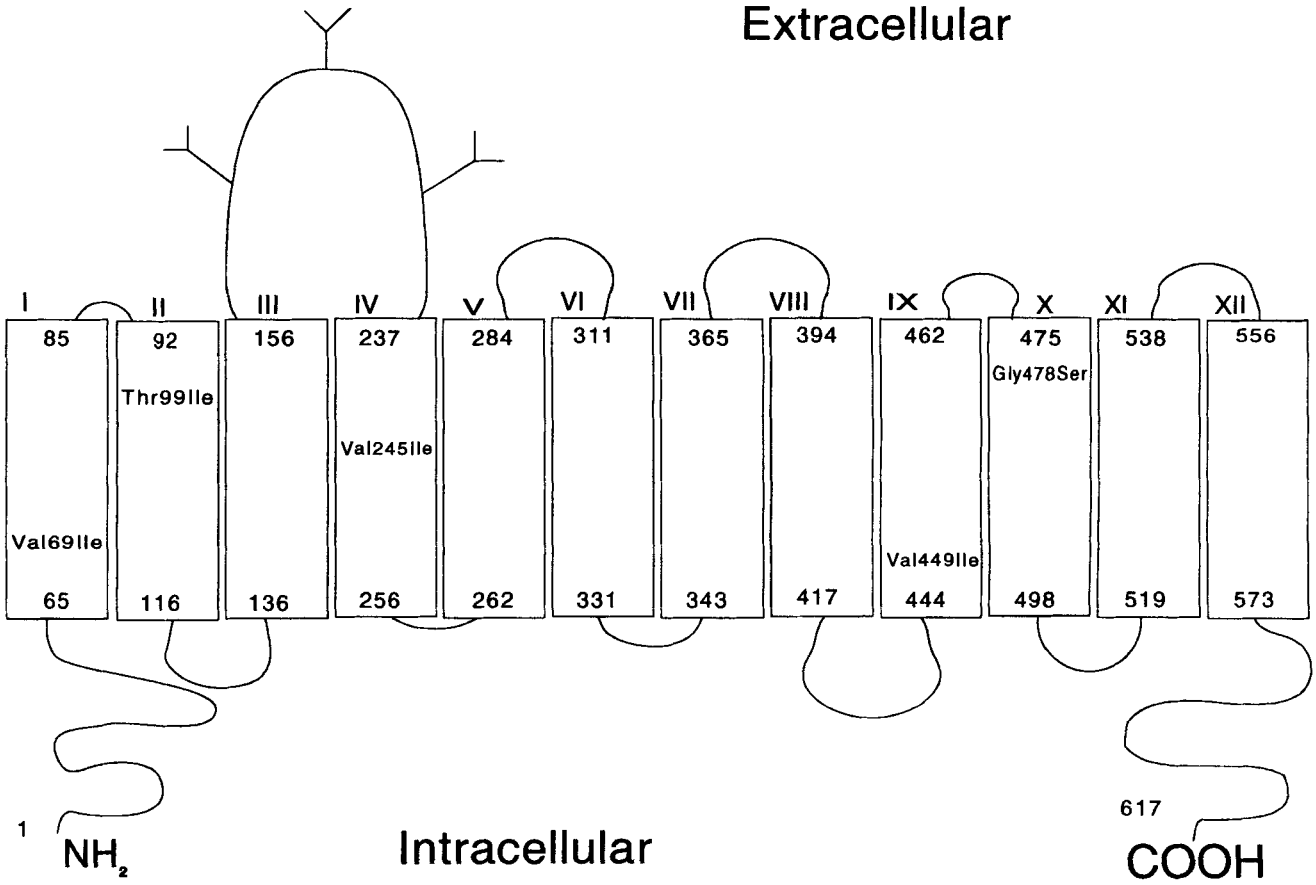

Fig. 3 Schematic representation of the human norepinephrine transporter gene and the location of naturally occurring amino acid substitutions.

Results from a case-control study indicate that presence of the genetic variants is not causally related to the development of bipolar affective disorder and schizophrenia. It remains a matter of future studies to delineate the relevance of these missense substitutions to transporter function and regulation and to investigate their possible association with other disease phenotypes or with individual differences in pharmacoresponse or occurrence of side effects [Propping and Nöthen, 1995].

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